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RNA Polymerase (J. Krakow, W. Horsley, M. Karstadt, M. Fine, R. Siegel)

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When RNA polymerase binds to DNA, it most probably undergoes conformational changes. This can be shown by studying the stability of the enzyme at elevated temperatures in the presence and absence of DNA. When RNA polymerase is heated in buffer at pH 8.2 for 2 minutes at 52°, there is a loss of 98% of the enzymatic activity and also the ability to bind sRNA. However, when the enzyme is incubated at 55° for 2 minutes in the presence of DNA, only 80% of the activity is lost. Therefore, enzyme bound to DNA is less sensitive to heat denaturation. There is no effect of addition of the ribonucleoside triphosphate substrates on the stability of the enzyme at this temperature. Both native and denatured calf thymus DNA protect the enzyme against heat denaturation. Polydextran sulfate which inhibits RNA synthesis by binding to the enzyme does not stabilize polymerase. There is no difference in the sensitivity of the DNA-enzyme complex heated at 47° or 50° for 5 minutes (50% loss of activity); enzyme alone heated at 47° for 5 minutes results in 50% loss of activity, while heating 5 minutes at 50° results in 90% loss of activity. These results suggest that polymerase "melts out" at a temperature between 47° and 50°. In order to gain further insight into the nature of the heat stability of RNA polymerase, were are presently purifying the enzyme from the thermophile, B. stearothermophilus.

Mechanism of Initiation and Termination of Peptide Chain Synthesis: Nucleotide sequences near the 5'-end of messenger RNA (M. Takanami, T. Okamoto, S. Goolsby, J. Levitt)

As described in the preceding Progress Report, we have succeeded in purifying polynucleotide kinase free from ribonuclease (RNase). This catalyzes the transfer of orthophosphate to the 5'-OH of polynucleotides. Using this enzyme, we were able to label the 5'-end of f2 RNA as a model

messenger RNA. However, it was noted that efficiency of labeling was very low even under optimal conditions. When an alkaline hydrolysate of f2 RNA which had been uniformly labeled with P^{32} was subjected to chromatographic analysis, a very small, but significant, radioactivity (less than 0.1%) was found in a mono-nucleotide polyphosphate region (tri- and tetra-phosphates). From these observations, we assumed that f2 RNA had a phosphate group or groups esterified on its 5'-end.

An alkaline phosphatase (APase) free from RNase was prepared by inducing APase in an RNase 1-deficient mutant, E. coli A19. When f2 RNA was treated with APase, the efficiency of terminal-labeling increased markedly. Alkaline and pancreatic RNase hydrolysates of f2 RNA, treated with APase and labeled with P^{32}, were subjected to chromatographic analyses. In addition, various mononucleotide-diphosphates and dinucleotide-triphosphates, as markers, were synthesized to identify the fragments from f2 RNA.

AU was identified as the main chain terminal sequence of f2 RNA. Analyses of further sequences are in progress.

Base Sequences in Nucleic Acids (S. Mandeles, F. Fearney, M. Kamio)

I. Sequence of bases of 5' linked end of TMV-RNA

A number of attempts were made to label the 5'-linked end of TMV-RNA with reagents such as 2, 4 dinitrophenylhydrazine, semicarbazide-C^{14} and sodium borohydride-H^3. The two main problems encountered in these procedures were the instability of the labeled RNA to subsequent isolation procedures (2, 4 dinitrophenylhydrazone), or non-specific labeling during the reaction ($NaBH^3$). To date, the most promising reagent has been semicarbazide-C^{14}. Briefly, the procedure involves oxidation of purified TMV-RNA with periodic acid to form TMV-RNA dialdehyde. This is then reacted with semicarbazide-C^{14} to form the bis-semicarbazone-C^{14} (SC^{14}) of TMV-RNA. After removal of unreacted semicarbazide, the labeled TMV-RNA-SC^{14} is hydrolyzed with T1 ribonuclease. The oligomers are separated according to chain length in an A-25-7 M urea system and all fractions are sampled for radioactivity. In a preliminary experiment, 90% of the total radioactivity was present in the $Gp^{=}$ peak. The inference is that there is a guanine in the fourth position at the 5'-linked end, and

the end piece consists of $X\tilde{p}Y\tilde{p}Z\text{-}SC^{14}$. However, since the initial separation was done at pH 5 it is likely that the separation did not occur strictly according to chain length, and that the $Gp^{=}$ peak contained small amounts of oligomers. This was borne out in a subsequent fractionation of the radioactive $Gp^{=}$ peak, according to base composition (AG 1x2 columns). Here the pattern showed numerous smaller peaks in addition to the very large $Gp^{=}$ peak, indicating possible degradation of contaminating oligomers. The radioactivity appeared in three small peaks. From the position in the chromatogram and from spectrophotometric base composition analysis, the radioactivity appeared to be in peaks corresponding to: adenosine or $A\text{-}SC^{14}$, ApA or $ApA\text{-}SC^{14}$, and Uridine or USC^{14}. It is interesting to note that the position of the last peak mentioned corresponds as well to $UpApA\text{-}SC^{14}$.

These experiments are being repeated with additional care to 1) assure initial separation according to chain length, and 2) minimize degradation during the preparation of samples for separation by composition.

II. Isolation and characterization of unique oligomers from TMV-RNA

(with D. Lloyd and I. Tinoco, Jr., Department of Chemistry)

When oligomers from T_1 ribonuclease hydrolysates of TMV-RNA are separated according to chain length (A-25-7M urea), the chromatography pattern shows a small peak at the end of, and well separated from, the main material. From its position, we estimate the chain length to be about 19. Preliminary base composition analysis indicates a chain length of at least 15. On the basis of a chain length of 19, the amount of material in this peak indicates the presence of a single oligomer per molecule of TMV-RNA.

The other long chain oligomers from the initial separation have been chromatographed according to base composition. Fractions from these separations have been rechromatographed according to chain length and again there are indications of unique oligomers (one per molecule of TMV-RNA) that can be isolated from the longer chain lengths.

Thymine Metabolism in E. coli (H. Kammen, E. Turner, M. Strand)

I. Thymidine phosphorylase: purification and mechanism of action

This enzyme has been highly purified from derepressed revertants of E. coli B_3 by osmotic and chromatographic procedures. The enzyme is bifunctional (Zimmerman, J. Biol. Chem., 239: 2622, 1964), catalyzing the reversible phosphorolysis of thymidine or deoxyuridine, as well as a direct deoxyribosyl transfer between these nucleosides and thymine or uracil. The transfer reaction is totally dependent on the presence of phosphate or arsenate, but does not involve the participation of deoxyribose-1-phosphate, which is not hydrolyzed or arsenolyzed.

The purified enzyme has a sedimentation constant of approximately 5.2 S as measured by sedimentation in sucrose; phosphorolytic and transfer activities both sediment together. No association or dissociation of the 5.2 S structure occurs under assay conditions, or in phosphate buffer or 2×10^{-4} M deoxyribose-1-phosphate.

The phosphorolytic activity does not involve participation of an enzyme phosphate ester group, since it is unaffected by treatment of the enzyme with alkaline phosphatase. Phosphorolysis probably involves a -SH group, however, since the enzyme is inhibited by p-hydroxymercuribenzoate and the inhibition is reversible by 10^{-2} M mercaptoethanol. These findings should permit further analysis of the subunit structure of the enzyme.

II. A new assay for thymidylate synthetase

A rapid and sensitive assay for thymidylate synthetase has been developed, based on the finding (Smith and Greenberg, Fed. Proc. 23: 271, 1964) that the hydrogen atom at carbon 5 of deoxyuridylic acid is released as a proton during the formation of thymidylic acid. The assay is carried out with dUMP-5-H^3 and is terminated by addition of Norit A, which absorbs the dUMP-H^3 but not the protons released. The Norit is removed by membrane filtration and the released tritium is measured directly from the filtrate.

The release of tritium shows an absolute requirement for tetrahydrofolate and formaldehyde and is optimal in the presence of Mg^{++}. These requirements are identical with those of purified thymidylate

synthetases, and are readily detectable in dialyzed crude extracts of E. coli B.

The rate of tritium release has been compared with the rate of dihydrofolate formation during phage T_4 infection of E. coli B. Extracts from both uninfected and infected cultures show a rate of tritium release 35% that of dihydrofolate synthesis. The reasons for this discrepancy are unclear but may be the result of isotope effects, as suggested by Smith and Greenberg.

III. Thymidylate synthetase activity in revertants of E. coli B_3-

A number of these revertants have been tested to determine whether thymidylate synthetase activity is regained during the restoration of prototrophy. Surprisingly, almost all of the revertants showed little or no activity when assayed with the method described above. The lack of activity is not due to the presence of inhibitory material in the extracts, nor to unusual heat lability of the enzyme. DNA isolated from revertant strains contain a normal complement of thymidylic acid. These findings suggest that an alternative pathway for synthesis of thymidine nucleotides may exist in these strains, or that the thymidylate synthetase of these organisms possesses unusual properties distinct from those of E. coli B.

Photo-Inactivation of TMV-RNA with Thiopyronin or Proflavin (B. Singer)

A comparison of the Diener method (B) (Virology 16: 140, 1962) with ours (A) at three time periods (6-17 hrs., 24 hrs., and 48 hrs.) showed that at all time periods more infectivity was found in the juice prepared by method A than B. However, all juices, whether prepared by methods A or B, reconstituted with TMV protein to a material which was 10-50X more infective than the juice. Both A and B juices were ultracentrifuged and yielded a pellet which reconstituted 20-60X and was 10-30% as infective as the original juice.

Both types of juices were treated with pancreatic ribonuclease under conditions where TMV is fairly stable, but TMV-RNA is completely destroyed. About the same retention of infectivity was found in the juices as in a comparable amount of TMV. However, little or no increase in infectivity was obtained by reconstitution indicating that most of the infectivity in the juice alone is viral and that the reconstituting portion is RNase-sensitive.

When the pelleted juice is fractionated in a sucrose gradient which gives clear separation of various components such as virus, infectious RNA, protein, and small molecular RNA, the reconstitutable material is throughout the gradient, including the gradient fractions which contain large amounts of plant RNA (probably 4S). Therefore, one must surmise that this material has variable densities, due perhaps to the presence of varying amounts of an additional plant material such as a lipid.

We believe that in the process of replication a virus-like material is present which is of low infectivity, partially RNase-resistant, capable of forming stable infectious TMV on the addition of TMV protein in 0.1M pH 7 pyrophosphate, and possibly associated with another complement altering its sedimentation properties.

Studies with Thermolysin (H. Matsubara, A. Singer, R. Sasaki)

Specificity study with thermolysin using synthetic peptides

In order to establish the detailed specificity of thermolysin, several synthetic peptides and their derivatives were used as the substrates of thermolysin. Leucinamide, Acetylleucine, Glycyl-phenylalanine, Glycyl-Proline, Prolyl-Alanine, Glycyl-Aspartic acid and Carbobenzoxy-Glycyl-Glycinamide were not hydrolysed to a measurable extent. Carbobenzoxy-Glycyl-Phenylalanine and Carbobenzoxy-Glycyl-Leucine were hydrolysed slowly. Substrates with no free α-carboxyl-group were hydrolysed very rapidly, including Carbobenzoxy-Glycyl-Phenylalaninamide and Carbobenzoxy-Alanyl-Leucine-hydrazide (3×10^{-1} μmole/mg protein/min. at 40° and pH 8 for the first-named substrate). These results agree very well with those obtained by protein digestion experiments. Thermolysin seems to be repelled by free amino and carboxyl groups near the sensitive peptide bond. Other substrates are being studied similarly.

Sequential Studies of Spinach Ferredoxin (H. Matsubara, R. Sasaki, A. Singer)

(In collaboration with the Department of Cell Physiology)

I. Exclusion of the possibility of β-elimination of cysteine residues in spinach ferredoxin as the source of labile sulfides

The H_2S evolved from Clostridial ferredoxin was suggested to result from β-elimination of cysteine residues in the the protein (Bayer et al., Archiv der Pharmazie 298: 196, 1965). After careful removal of iron from spinach ferredoxin, it was oxidized, hydrolysed, and analysed. The cysteic acid content of iron-free ferredoxin was exactly the same as that of ferredoxin from which H_2S had evolved as a result of trichloroacetic acid treatment. Therefore, the possibility of β-elimination of cysteine residues was excluded.

II. Use of modified Edman's PTC (phenylisothiocyanate)-method on carboxymethyl-ferredoxin

Edman's three-step PTC method was modified slightly and applied to carboxymethyl-ferredoxin. The results showed excellent recovery of phenylthiohydantoyl-amino acids and so far 5 steps were performed without any significant internal peptide bond cleavage. The amino terminal sequence of spinach ferredoxin was found to be Ala-Ala-Tyr-Lys-Val-.

III. Tryptic peptides of aminoethylated ferredoxin

Twenty-six fractions were obtained from tryptic digests of aminoethylated ferredoxin and the identification of several peptide sequences was completed. Some are very interesting in terms of the study of the comparative structure and active centers of the homologous proteins of the ferredoxin series.

Studies on DNA Replication in Bacillus subtilis (H. Yoshikawa, B. Benjamin, E. Cook, M. Haas)

I. DNA-protein complex isolated from B. subtilis

We have previously reported isolation of a stable DNA-protein complex from the cell membrane fractions of B. subtilis. A large amount of the complex was purified, first by sucrose gradient centrifugation, and then by CsCl gradient centrifugation. The purified complex was hydrolyzed with 6N HCL in a sealed tube at 105° for 20 hours and analyzed for its amino acid composition. This analysis was kindly carried out by Dr. H. Matsubara.

The results are shown in Table I. Data indicating the amino acid composition of the total protein and histone-like protein from B. subtilis are also shown. The molar ratio of nucleotide residues to amino acid residues in the complex was 2.9. The serine content was remarkably high, suggesting that serine is important in the interaction between protein and DNA. However 8-hydroxyquinoline released about 50% of the protein from the complex suggesting that metals may have a role in the binding of protein to DNA.

TABLE I

Amino Acid Composition of the Stable DNA-Protein Complex

	Protein of the stable DNA-protein complex	Histone-like[1] protein	Total protein[2]
Arginine	3.9	7.7	4.6
Histidine	3.3	1.0	2.4
Lysine	11.3	7.4	8.0
Aspartic acid	8.7	15.0	11.3
Glutamic acid	13.2	11.2	13.0
Serine	20.7	8.7	5.6
Threonine	6.5	6.3	6.5
Tyrosine	1.7	4.0	3.2
Methionine	2.0	0	2.9
Methionine-sulfoxides	--	1.2	--
Cystine	0.6	0	--
Alanine	10.1	11.2	10.0
Glycine	--	--	--
Isoleucine	3.2	5.9	5.6
Leucine	4.6	9.6	9.4
Phenylalanine	2.6	4.7	4.3
Proline	2.5	Trace	4.8
Valine	5.0	6.1	8.4
Total	99.9	100.1	100.1

[1] From N. V. Bhagavan and W. A. Atchley, Biochem. 4: 234, 1965.

[2] From N. Sueoka, Proc. Nat. Acad. Sci. 47: 1141, 1961.

II. DNA replication in vitro

We reported previously that the particulate fraction of schock-treated protoplasts ("PFSP") of B. subtilis could carry out DNA synthesis in vitro. (Progress Report, Series No. 6, Issue No. 12, page 51). Some of the characteristics of this system were also reported in Proc. Nat. Acad. Sci. 53: 1475, 1965.

At that time we did not characterize the in vitro products in terms of density because of the difficulty of obtaining spores labelled with heavy isotopes such as D_2O and ^{15}N.

In order to obtain a heavy density DNA from an in vitro system, we have tried to use 5-bromo-deoxyuridine-5'-triphosphate (BudUTP) in place of deoxythymidine-5'-triphosphate (TTP). BudUTP was synthesized from deoxyuridine-5'-triphosphate by bromination and was purified by column chromatography. DNA polymerase was partially purified from B. subtilis by the method of Okazaki and Kornberg.

When purified DNA was used as a primer, BudUTP was incorporated 80% as fast as was TTP. Preliminary results showed that a heavy DNA was synthesized by the PFSP with BudUTP as a substrate. Comparisons of in vitro reactions between purified enzyme and PFSP prepared from spores germinated under various conditions are now under investigation.

Optical Properties of Viruses and Their Nucleic Acids (M. Maestre, K. Sieux)

I. Lambda bacteriophage density mutants b_1 and b_5 were purified in large enough quantities to allow the measurement of the optical properties such as optical rotatory dispersion (O.R.D.) and absorption spectra of the intact virus.

II. The optical behavior of nucleic acids in very high salt solutions is being studied following the techniques of Geiduschek (J. Mol. Biol. 4: 467, 1962) and Hamaguchi and Geiduschek (J. Amer. Chem. Soc. 84: 1329, 1962) since the behavior of nucleic acids in solutions of very low water activity mirrors the state of the molecule as it is found in the virus coat protein.

III. All the optical activity curves of the viruses that have been studied so far have been analyzed. The main conclusion is that the effect of the packing of the nucleic acid in the head of the virus is to change the O.R.D. spectra by superimposing a single Cotton effect curve centered about the 260mμ absorption band. A manuscript describing this research is being prepared for publication. It will discuss the meaning of this Cotton effect in relation to the internal packing of the nucleic acids in viruses.